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Development of an Enzyme-Linked Immunosorbent Assay for Detection of the Gregarious Hymenopteran Parasitoid *Muscidifurax raptorellus* in House Fly Pupae

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***Muscidifurax raptorellus* (Kogan and Legner) are gregarious parasitoids of stable fly (*Stomoxys calcitrans* L.) and house fly (*Musca domestica* L.) puparia. Strategic inundative releases of mass-reared *M. raptorellus* and other microhymenopteran parasitoids have been used in studies of biological control of pest and hemophagic dipterans that develop in livestock waste. Lack of an accurate and simple method to estimate pupal parasitism rates has limited the assessment of the utility of filth fly biological control by *M. raptorellus* and other parasitoids. Conventional estimates of parasitism rates are based on measurements of both the emergence of adult wasp parasitoids from puparia and the noneclosion of adult host flies (assuming parasitoid-induced pupal mortality). Microdissection of dipteran pupae for parasitoid presence is a difficult and tedious alternative method to estimate parasitism rates. In the present study, we produced polyclonal rabbit anti-*M. raptorellus* larvae serum antibodies, absorbed the antiserum with house fly puparia homogenates to remove muscoid host-reactive antibodies, and used the absorbed antiserum in a rapid enzyme-linked immunosorbent assay (ELISA) to detect *M. raptorellus* larvae in parasitized house fly pupae. The ELISA accurately detected *M. raptorellus* parasitism of house fly pupae between 7 and 21 days poststing and detected parasitoids in nonemergent house fly pupae. Absorbed antiserum specificity for parasitoid antigens was confirmed by Western blots. This first report of muscoid pupal parasitoid detection by enzyme immunoassay suggests that this approach has potential as a research and surveillance tool for monitoring and quantifying the success of parasitoid releases for biological control of dipteran pests.**

***Key Words: Muscidifurax raptorellus; Muscidifurax raptor; Spalangia nigroaenea; Musca domestica; Stomoxys calcitrans;* gregarious microhymenopteran pupal parasitoid; biological control; enzyme-linked immunosorbent assay.**

INTRODUCTION

Parasitic wasps of the genus *Muscidifurax* (Hymenoptera: Pteromalidae) are pupal parasitoids of synanthropic Diptera, including house flies and stable flies, which have been used as biological control agents for livestock dipteran pests. Whereas most species of *Muscidifurax* oviposit a single egg on each host pupa (solitary oviposition), *M. raptorellus* (Kogan and Legner) practice gregarious oviposition in which multiple ova can be deposited on each host pupa. This species has demonstrated promise as a biological control agent against house fly and stable fly pupae in several studies (Petersen and Cawthra, 1995; Petersen and Currey, 1996a; Floate *et al.*, 2000). An aggressive stinging behavior along with its gregarious nature make *M. raptorellus* relatively easy to mass rear for inundative parasitoid releases (Petersen and Currey, 1996b).

The most common method for estimating pteromalid parasitism rates in dipteran pupae in biological control studies is by the measurement of adult parasitoid emergence in field-collected or sentinel (laboratory-reared) host pupae (Petersen *et al.*, 1992; Andress and Campbell, 1994; Petersen and Cawthra, 1995; Weinzierl, and Jones, 1998). However, the longer life cycle of pteromalids (compared to Diptera) and the occurrence of diapause prior to adult parasitoid emergence are drawbacks to this conventional approach. Pupal microdissection for parasitoid detection is a tedious and time-consuming alternative to parasitoid progeny emergence. The frequent occurrence of noneclosed host pupae from which adult parasites do not emerge

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("duds") (Weinzierl and Jones, 1998) in pteromalid biological control studies is a confounding issue and a cause of misclassification bias. The common assumption that all host pupal noneclosion is due to parasite-induced mortality inflates true parasitism rates by an unknown factor, whereas exclusion of all duds underestimates actual parasitism rates since true parasitoid-induced mortality in noneclosed host pupae is missed altogether. Our goal in the present study was to develop an accurate (sensitive and specific), inexpensive, and immediate (near real time results) enzyme-linked immunosorbent assay (ELISA) to measure pteromalid parasitism rates in livestock pest dipteran pupae as an ecologically useful tool for use in future biological control studies. In particular, we wanted to develop an immunoassay which would be able to detect *M. raptorellus* in host pupae across the spectrum of various larval ages and in nonemergent, noneclosed but parasitized host pupae so that misclassification bias would be minimized.

MATERIALS AND METHODS

Parasitoids and Host Diptera

Hymenopteran parasitoids (gregarious: *M. raptorellus;* nongregarious: *Spalangia nigroaenea* (Curtis) and *Muscidifurax raptor* (Girault and Sanders)) and dipteran hosts (*Musca domestica* L. and *Stomoxys calcitrans* L.) were laboratory reared using previously described methods (Morgan, 1985). Stock host pupal antigens were prepared by maceration of the internal contents of unparasitized house and stable fly pupae in phosphate-buffered saline (PBS, pH 7.4). Parasitoid antigens were prepared by careful dissection of *M. raptorellus* larvae from house fly host pupae and maceration of the wasp larvae in PBS. A stock *M. raptorellus* antigen was prepared by the pooling of 7-, 14-, and 21-day-old parasitoid larvae. In addition, *M. raptorellus* larvae parasitoid antigen preparations of age 3–5, 7–8, 14, and 21 days were also prepared by the pooling of microdissected larvae. Protein concentrations of host and parasitoid antigen preparations were determined by use of a modified Lowry assay (BCA Protein Assay; Pierce, Rockford, IL). In addition, the protein concentrations of a sample of individual unparasitized host *M. domestica* and *S. calcitrans* whole pupae were determined.

Preparation of Parasitoid Immunizing Antigen and Rabbit Immunization Protocol

Larval *M. raptorellus* from 7 to 21 days old were harvested from parasitized laboratory-reared house fly pupae by careful microdissection which minimized contamination of parasites with host tissues. The parasitoids were suspended in PBS at about 1 g parasites/ml and finely homogenized for 15 min in a 10-ml glass tube on ice by use of a tissue grinder. The homogenized parasite suspension was then centrifuged at 10,000*g* for 20 min at 4°C. The protein concentration of the antigen preparation supernatant was determined and the antigen was stored at −20°C until use. Four 12-week-old New Zealand white rabbits were randomly allocated into control and immunization groups. The two control rabbits were not immunized. On day 0, the two rabbits selected for immunization were injected intramuscularly with 0.4 ml of an emulsion of 4 mg protein/ml of the parasite antigen in complete Freund's adjuvant (Sigma, St. Louis, MO). On day 29, the rabbits were given an intramuscular booster injection of 0.4 ml of an emulsion of 4 mg protein/ml of the parasite antigen in incomplete Freund's adjuvant (Sigma). On day 57 a final intravenous booster injection of 0.5 ml of 2 mg/ml parasite antigen (without adjuvant) was given in the marginal ear vein. On day 67, immunized rabbits were euthanized and their blood was collected. The rabbit serum was separated from the blood cells by centrifugation at 500*g* for 20 min at 4°C. Serum samples were also collected from all rabbits at day 0 (pre-immunization sera) and at day 57. Serum was frozen at −20°C until use.

Absorption of Polyclonal Antiparasitoid Antiserum with House Fly Pupal Antigen

Approximately 100 unparasitized house fly pupae were macerated in 1.0 ml of PBS in a microcentrifuge tube. The tube was centrifuged twice for 10 min at 10,000*g* and the pellet discarded. The protein concentration of the remaining suspension was then determined as described above. Five milliliters of immunized rabbit sera was then mixed with 850 μl of house fly antigen suspension (100 mg total protein/ml) in a 10-ml plastic tube and absorbed (incubated) in a 37°C water bath for 2 h with occasional manual agitation. The tube was then centrifuged at 500*g* for 15 min, which produced a small visible pellet (presumably composed of a precipitate of antigen–antibody complexes). The absorbed serum (supernate) was saved, and the pellet was discarded. Two percent sodium azide solution (Sigma) was added (10 μl/ml of volume) to the absorbed serum as a preservative.

Parasitoid ELISA Development

Initial ELISA working conditions were defined by use of checkerboard dilutions of negative (dipteran pupae) and positive (*M. raptorellus* larvae) control antigens (range 1 to 100 μg/ml), primary antibody (unabsorbed and absorbed immune rabbit sera), and horse radish peroxidase (HRP)-labeled secondary antibody (goat anti-rabbit IgG-HRP conjugate). Antigen coating times and antibody incubation times were also varied. After initial assay conditions were discovered [i.e.,

where (positive) signal-to-(background) noise ratio was greatest for parasite antigen and background ELISA absorbance values were low for negative host antigens], the ELISA was further refined and optimized by comparison of ELISA results with absorbed and unabsorbed sera, by varying of the time of substrate (ABTS) development, and by coating of the plates with known parasitized and unparasitized whole house fly pupae.

Optimized Parasitoid ELISA Conditions

A modification of a rapid indirect ELISA procedure (He *et al.*, 1996) was used. Ninety-six-well disposable flexible polyvinyl chloride round "U"-bottom microtiter plates (Catalog No. 2101; Dynatech Laboratories, Inc., Chantilly, VA) were coated either with pure parasitoid, house fly, or stable fly pupal antigen preparations or with macerated stung or unstung pupae diluted in carbonate coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6). After being covered with seal-tape (ELISA sealing tape; Corning Costar Corp., Cambridge, MA), the plates were incubated for 2 h at 37°C. Coated plates were either used immediately or stored at −20°C until use.

Pupal antigen-coated plates were washed five times with ELISA wash buffer (phosphate-buffered saline [PBS]–Tween–horse serum; consisting of 0.923% fluorescent treponemal antibody (FTA) hemagglutination buffer powder (PBS; Becton–Dickinson, Cockeysville, MD)), with 0.1% Tween 80 (Sigma), and with 0.5% sterile filtered horse serum (Sigma) by use of an automatic 96-well plate washer (EL404 Microplate Autowasher; Bio-Tek Instruments, Inc., Winooski, VT). Postimmunization-absorbed rabbit sera (1:2400 dilution in wash buffer) was added (100 μl) to each well. The plates were then incubated for 10 min at 37°C. After being washed five times, goat anti-rabbit IgG horse radish peroxidase (HRP)-labeled antibody [Kirkegaard and Perry Laboratories (KPL), Gaithersburg, MD] (1:1000 dilution in wash buffer) was added to each well (100 μl) and the plates were incubated for another 10 min at 37°C. After being washed five times, 100 μl of a commercial substrate solution [0.01% H_2O_2, 2,2′-azino-di (3-ethyl-benzthiazoline sulfonate (6))] in glycine/citric acid buffer (ABTS one-component peroxidase substrate; KPL) was added to each well. The enzymatic color reaction was allowed to proceed for 15 min at room temperature and stopped by the addition of 50 μl of 1% sodium dodecyl sulfate (SDS) per well. The absorbance in optical density (OD) units of each well was read at dual wavelengths of 405 and 490 nm with an automated ELISA reader (EL311 Microplate Autoreader; Bio-Tek Instruments). The reading of plates at dual wavelengths (405 nm test/490 nm reference) is a normalization procedure that generates more accurate absorbance readings (compared to single-wavelength readings) by removing background optical density noise unrelated to the substrate reaction (Richardson *et al.*, 1983). Coating buffer control, HF, SF, and parasitoid antigen (10 μg/ml) were included on each plate run. All control and test samples were run in duplicate on each plate.

ELISA Assessment: Negative- and Positive-Reference Pupae

Following initial optimization of ELISA conditions by use of a small number of known parasitized and unparasitized house fly pupae, a panel of 201 negative-reference (unparasitized) host dipteran pupae (101 house fly, 100 stable fly) and 427 microdissection-verified positive-reference (*M. raptorellus* parasitized) house fly pupae with larvae of various developmental (postoviposition) ages was tested by ELISA to assess the optimized ELISA performance and to determine an appropriate assay cutoff value. Parasitized house fly pupae included 1–3, (n = 50), 4–5, (n = 50), 7, (n = 107), 14, (n = 110), and 21 days (n = 110) postexposure to *M. raptorellus* oviposition. All positive control parasitized house fly pupae contained at least one *M. raptorellus* larva (mean of 5 and range of 1–10 parasitoids per host pupa). Both the negative and the positive control house fly pupae were macerated with a pellet pestle in 100 μl PBS in a 1.5-ml microcentrifuge tube, further diluted with 900 μl of PBS, and vortexed. This resulted in a stock concentration of about 17.5 mg total protein/ml. Next, 9 μl of the stock pupal solution was added to 1.5 ml of carbonate coating buffer in a 5-ml glass tube to produce a host pupae or a host pupae–parasitoid solution of about 100 μg protein/ml. Each 100-μg protein/ml solution was assayed by the ELISA described above.

Western Immunoblotting Assay

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western immunoblotting procedures were performed as previously described (Westerman *et al.*, 1997) with minor modifications. Whole *M. raptorellus* parasitoid, house fly pupae, and stable fly pupae antigen preparations were identically loaded (20 μg protein/well) onto discontinuous SDS–polyacrylamide (5% stacking and 10% separating) gels. After electrophoresis, one gel was stained with Coomassie brilliant blue R-250 and the antigens in the other gels were transferred (100 V for 2 h at 4°C in 0.2% 3-[cyclohexylamino]-1-propane sulfonic acid (CAPS) buffer with 10% methanol) onto polyvinylidene difluoride (PVDF) membranes (Millipore Immobilon-P, Burlington, MA). The membranes were blocked in Tween Tris-buffered saline (TTBS; 10 mM Tris–HCl [pH 7.6], 0.9% NaCl, 0.05% Tween 20) (Sigma) with 5% nonfat dry milk (Carnation) overnight at 4°C and then probed with the absorbed and unabsorbed polyclonal rabbit anti-*M. raptorellus* serum (diluted 1:1000 in

TTBS with 3% nonfat dry milk) for 1.5 h at 22°C. Following three 5-min washes in TTBS with 3% nonfat dry milk on a shaker, the membranes were incubated with the HRP-conjugated goat anti-rabbit IgG (1:1000 in TTBS with 3% nonfat dry milk) for 1 h at 22°C. Following three 5-min washes in TTBS with 3% nonfat dry milk and a 5-min rinse with TTBS (without milk), the substrate diaminobenzidine (DAB; KPL) solution (2% DAB and 0.01% H_2O_2 in 0.1 M Tris [pH 7.6]) was used to visualize specific reactive protein bands. The DAB colorimetric reaction was stopped by the rinsing of the membranes in distilled water after 2.5 min development time.

ELISA Validation: Blinded Comparison of Parasitoid ELISA with Microdissection-Verified Standards

The ELISA diagnostic performance was evaluated on a second set of 263 muscoid pupae to validate assay performance after cutoff selection. Unstung house fly ($n = 109$) and microdissection-verified *M. raptorellus*-parasitized house fly pupae of 7–8 ($n = 55$), 14 ($n = 49$), and 21 days ($n = 50$) were macerated and diluted in 1 ml of PBS and given a number code. The samples were then tested blindly (i.e., without knowledge of the parasitized or nonparasitized status of the pupae) by ELISA as described above and classified as parasitized or not using the threshold absorbance cutoff determined from the negative- and positive-reference pupae. Results from ELISA were then statistically compared to microdissection results.

Parasitoid ELISA Protein Detection Limit

Microtiter plates were coated with whole house fly and 4- to 5-, 7- to 8-, 14-, and 21-day-old *M. raptorellus* parasitoid antigens at serial twofold-diluted total protein concentrations ranging from 100.0 to 0.08 μg/ml. The ELISA was then run on these samples under the optimized conditions to determine the parasitoid larval age-dependent detection limits of the assay. The ELISA protein detection limit for each antigen preparation was defined as the smallest coating antigen concentration that gave an absorbance value above the assay cutoff value.

Artificial Nonemergent House Fly Pupae

A total of 200 viable house fly pupae were exposed to *M. raptorellus* adult wasps for 48 h and allowed to develop for 14 days. A random subset of 100 of these pupae were rapidly freeze-killed by exposure to −20°C for 24 h and held at room temperature for 7 days. The other 100 wasp-exposed pupae were allowed to fully mature to emergence or eclosion to obtain an approximate expected parasitism rate for the freeze-killed pupae. The freeze-killed pupae were then tested by ELISA for presence of parasitoid antigens. The proportion of parasitized pupae by ELISA versus wasp emergence was then compared.

Non-M. raptorellus Pteromalidae

To estimate the phylogenetic specificity of the ELISA for *M. raptorellus* larval parasitoids as opposed to other pteromalids, the ELISA was also run on microdissection-verified house fly pupae containing nongregarious 10- to 14-day-old *M. raptor* ($n = 50$) and 10-day-old *S. nigroaenea* ($n = 20$).

Effect of Absorbed Versus Unabsorbed Immune Rabbit Serum

Microtiter plates were coated with whole house fly and stable fly pupal antigens and stock (pooled 7-, 14-, and 21-day-old larvae) *M. raptorellus* parasitoid antigen at serial twofold-diluted total protein concentrations ranging from 20.0 to 0.01 μg/ml. The ELISA was then run on these samples under the optimized conditions using both absorbed and nonabsorbed anti-*M. raptorellus* antisera to determine the effect of serum absorption on assay detection limit and specificity.

Data Analysis

For negative- and positive-reference pupae, dot-box plots (Krieg *et al.*, 1988) were created to visualize the point distributions of parasitoid larval age-specific ELISA absorbance values by use of commercial software (Prism 3.0; Graph Pad Software Inc., San Diego, CA). Receiver-operator characteristic (ROC) curve methodology (Zweig and Campbell, 1993) was used to determine the global discriminatory (accuracy) ability of the parasitoid ELISA and to aid in selection of the assay cutoff point. Receiver-operator characteristic curves and related statistics were created using commercial (True Epistat Version 3.1; Epistat Services, Richardson, TX) and shareware (Gorog, 1994) software. The ROC curve is a graph, in the unit square, of the (1-specificity, sensitivity) pairs that are formed as the cutoff value moves through its range of possible values (Beam and Weiand, 1991). The 1-specificity is equivalent to the false positive rate and the sensitivity is equivalent to the true positive rate. The ROC curve is monotone increasing and always lies on or above the 45-degree line. A test with perfect discrimination (i.e., no overlap between the negative and the positive distributions) has an ROC plot that passes through the upper left corner, where the true positive fraction is 1.0 (i.e., 100% sensitivity) and the false positive fraction is 0. The theoretical plot for no discriminatory ability (i.e., identical distributions for the negative- and positive-reference groups) is a 45-degree line ("the guess line") from the lower left corner at the origin to the upper right corner. Therefore, a test with an ROC curve coinciding with the diagonal is worthless and the

discriminatory power of a test increases as the ROC curve deviates upward and toward the left corner. The area under the ROC curve is advocated as an unbiased unit measure (index) of the overall diagnostic value of a test, with greater area indicating a better test (Linnet, 1988). The area under the ROC curve will vary between 0.5 for a test with no distributional differences between states and 1.0 for perfect separation of negative- and positive-reference conditions. A *z* score test statistic is calculated to compare the estimated ROC curve area with the null hypothesis that the ROC area equals 0.5. A *P* value (based on the *z* score) less than 0.05 indicates that the ROC area is statistically greater than 0.5 and that the diagnostic test has significant overall discriminatory ability (Hanley and McNeil, 1982; Beck and Schultz, 1986). The overlap index was also calculated as an additional method of global-level test performance (Hartz, 1984). The overlap index is a small-sample analog of the Wilcoxon rank-sum test and is a nonparametric measure of the degree to which the negative- and positive-reference states overlap. The index ranges from 0 for an ideal test with no overlap to 1 if the rank order of values is the same for the two states. The overlap index is related to the area under the ROC curve by the formula ROC area = 1 − (overlap index/2) (Zweig and Campbell, 1993).

Our ELISA performance goal was development of an assay with the highest possible sensitivity (across the spectrum of postoviposition *M. raptorellus* larval parasitoid ages) and 100% diagnostic specificity, i.e., without muscoid host cross-reactivity. The dot-box plots and ROC curves were used to select an ELISA cutoff that achieved these assay performance characteristics. The cutoff value (decision threshold) is the ELISA absorbance value used to distinguish negative from positive pupae; i.e., all reference samples with $OD_{405/490}$ values at or above this threshold were considered "positive" and those below this value were considered "negative." After the ELISA cutoff value was determined, the sensitivity (No. ELISA-positive samples/No. positive-reference samples tested) and specificity (No. of ELISA-negative samples/No. negative-reference samples tested) with exact binomial 95% confidence intervals (Rosner, 1986) were calculated for the optimized parasitoid ELISA (at this cutoff) by use of public domain statistical software (Epi Info 6.02; Centers for Disease Control and Prevention, Atlanta, GA).

For the 263 microdissection-verified pupal samples on which the ELISA was performed blinded as to parasite status, the sensitivity, specificity, and agreement (with exact binomial 95% confidence intervals) were calculated. Agreement between microdissection and ELISA results was measured both by concordance (proportion of overall test agreement unadjusted for chance agreement) and by the kappa statistic (κ, the proportion of chance-corrected test agreement) (Fleiss, 1981). The McNemar's χ^2 test (with continuity correction) was used to determine whether microdissection and the ELISA provided statistically different test results (Courtney and Cornell, 1990). A χ^2 value corresponding to a P value >0.05 indicates no difference in test results in the compared diagnostic methods.

RESULTS

Optimized Parasitoid ELISA Conditions

The optimized ELISA conditions were the following: coating pupae concentration of 10 μg protein/ml, primary antibody concentration of 1:2400 with absorbed sera; secondary antibody concentration of 1:1000, and ABTS colorimetric development time of 15 min.

ELISA Assessment: Negative- and Positive-Reference Pupae

The distributions of the net absorbance values for the parasitoid negative- and parasitoid positive-reference fly pupae are shown in Fig. 1; the corresponding ROC curves are shown in Fig. 2. Based on the ELISA OD distributions, the ROC curve for all 201 negative-reference and 427 positive-reference pupae (Fig. 2, curve A), and our specified goal of an assay with the highest sensitivity possible at 100% specificity, the cutoff value (threshold) to distinguish negative from positive pupae was defined as the sample absorbance minus the buffer control absorbance (=net absorbance) plus 0.200 OD units. Mean and standard deviation (SD) ELISA absorbance values ($OD_{405/490}$) and estimates of diagnostic test specificity (overall and by dipteran host) and sensitivity (overall and by *M. raptorellus* larval age) with 95% confidence intervals for the parasitoid ELISA at the selected cutoff are summarized in Table 1.

With net absorbance plus 0.200 OD units as the positive cutoff, the *M. raptorellus* ELISA test had no false positive reactions among the 201 negative-reference house fly and stable fly pupae (100% specificity). As shown in Table 1 and Fig. 1, both the diagnostic sensitivity and the mean OD for positive-reference fly pupae increased with greater larval parasitoid age. The ELISA sensitivity was low for fly pupae containing *M. raptorellus* larvae less than 7 days old but was uniformly high for pupae containing parasitoid larvae 7 days old or greater (Table 1). Furthermore, the ELISA absorbance values were very high for pupae containing parasitoid larvae of 14 to 21 days of age.

The larval age-specific global performance of the *M. raptorellus* parasitoid ELISA was summarized and further quantified by the area under the ROC curve and the overlap index (Table 2). The area under the ROC curve increased and the overlap index decreased as the parasitoid larval age increased from 1 to 21 days of age. Figure 2 shows three ROC curves corresponding to (A)

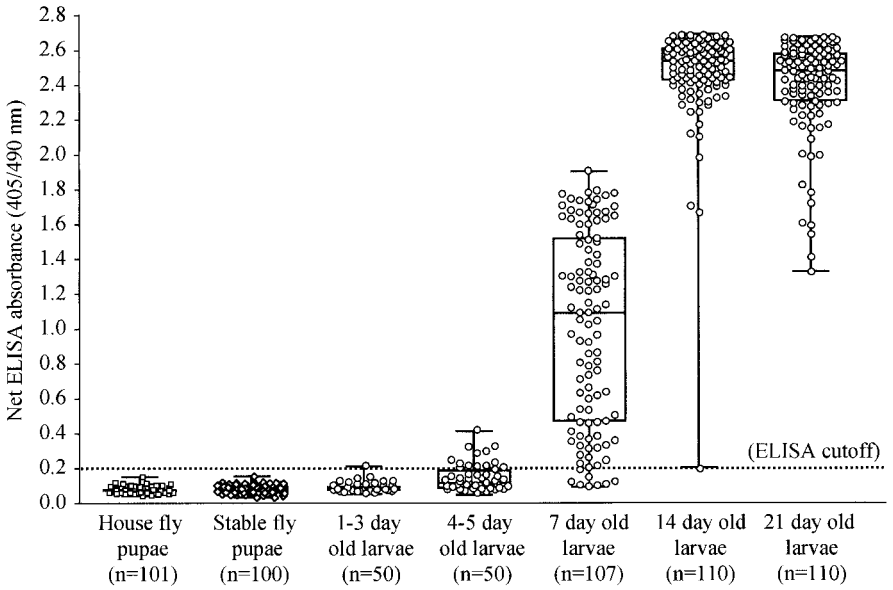

FIG. 1. Dot-box plots of net ELISA absorbance values for 201 negative-reference house fly and stable fly pupae and 427 positive-reference *M. raptorellus*-parasitized house fly pupae of various developmental ages. Dots represent mean $OD_{405/490\ nm}$ values of duplicate wells. On the superimposed box plots, bottom and top box edges mark the 25th and 75th distribution percentiles, the central horizontal line represents the median (50th percentile), and the central vertical lines extend from the box as far as the data extend (range).

all 201 negative-reference pupae and all 427 positive-reference pupae with larvae of 1 to 21 days of age, (B) all 201 negative-reference pupae and the subset of 327 positive-reference pupae with larvae of 7 to 21 days of age, and (C) 101 house fly negative-reference pupae and the subset of 100 positive-reference pupae with larvae of 1–5 days of age. These three ROC curves further demonstrate that the ELISA significantly discriminated nonparasitized dipteran pupae from those parasitized by *M. raptorellus* of all larval ages and that the ELISA diagnostic accuracy improved, essentially to perfection, as larval age increased.

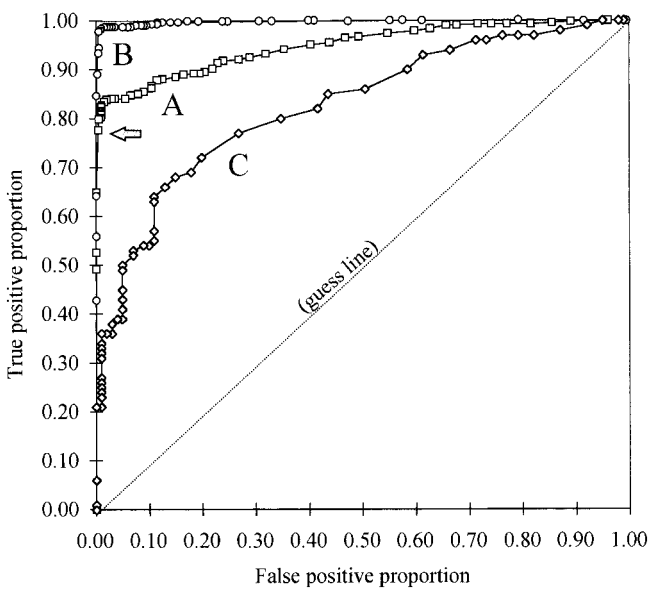

FIG. 2. Receiver-operating characteristic (ROC) curves for *M. raptorellus* ELISA. The ROC curves are derived from the dot-box plot data shown in Fig. 1. (A) Curve resulting from 101 house fly and 100 stable fly negative-reference pupae and 427 positive-reference house fly pupae with 1- to 21-day-old larvae; the arrow indicates the location of the ELISA cutoff (net $OD_{405/490\ nm} = 0.200$) on this curve; (B) curve resulting from 101 house fly and 100 stable fly negative-reference pupae and subset of 327 positive-reference pupae with 7- to 21-day-old larvae; (C) curve resulting from 101 house fly negative-reference pupae and 100 positive-reference pupae with 1- to 5-day-old larvae.

Western Immunoblotting Assay

The results of Western immunoblots using both absorbed and unabsorbed sera on house fly and stable fly pupae and on *M. raptorellus* parasitoid antigens of various larval ages are shown in Fig. 3. The areas of cross-reactivity among the host and parasitoid antigens tended to be proteins of higher molecular weight (>~50 kDa).

ELISA Validation: Blinded Comparison of Parasitoid ELISA with Microdissection-Verified Standards

The distributions of the net absorbance values for microdissection-verified parasitoid-negative ($n = 109$) and parasitoid-positive ($n = 154$) house fly pupae of various larval ages from 7 to 21 days that were tested blindly by ELISA following microdissection are shown in Fig. 4. The comparative performance and agreement between these two parasitoid detection methods is shown in Table 3. There were two false-negative and two false-positive ELISA results, corresponding to a sensitivity of 98.7% and a specificity of 98.2% for the

TABLE 1

Muscidifurax raptorellus (*MR*) ELISA Mean and Standard Deviation (SD) Optical Density ($OD_{405/490}$) and Diagnostic Specificity/Sensitivity for 201 Negative-Reference (Unparasitized) House Fly and Stable Fly Pupae and 427 Microdissection-Verified Positive-Reference (*M. raptorellus*-Parasitized Larvae) House Fly Pupae of Various Developmental Ages with Cutoff for Positivity at Net Absorbance of 0.200 OD Units

Pupal sample	Mean ELISA OD (SD)	ELISA-positive samples/ samples tested	Diagnostic estimate and confidence interval (CI)	
			Specificity/sensitivity (%)	95% CI
		Negative-reference		
House fly	0.0775 (0.0158)	0/101	100.0	96.4–100.0
Stable fly	0.0828 (0.0218)	0/100	100.0	96.4–100.0
All pupae	0.0802 (0.0192)	0/201	100.0	98.2–100.0
		Positive-reference house fly		
1- to 3-day-old *MR*	0.0963 (0.0275)	2/50	4.0	0.5–13.7
4- to 5-day-old *MR*	0.1548 (0.0794)	13/50	26.0	14.6–40.3
7-day-old *MR*	1.0125 (0.5554)	99/107	92.5	85.8–96.7
14-day-old *MR*	2.4693 (0.2806)	110/110	100.0	96.7–100.0
21-day-old *MR*	2.3983 (0.2789)	110/110	100.0	96.7–100.0
All *MR* pupae	1.5370 (1.0347)	334/427	78.2	74.0–82.0

parasitoid ELISA relative to microdissection. Both ELISA false-negative samples were from fly pupae with 7- to 8-day-old *M. raptorellus* larvae. The high level of diagnostic agreement between these two methods was confirmed statistically by the large kappa statistic ($\kappa = 0.969$) and the nonsignificant McNemar's χ^2 ($P = 0.617$).

Parasitoid ELISA Protein Detection Limit

The ELISA muscoid host and parasitoid protein detection limit titration curves and detection limits are shown in Fig. 5. The assay could detect as little as 0.31 and 0.63 μg protein/ml (0.031 and 0.063 μg per microplate well) of *M. raptorellus* antigen from 14- and 21-day-old pupae. The assay had much higher detection limits of 10 and 2.5 μg/ml for *M. raptorellus* antigen derived from younger larvae of 4–5 and 7–8 days of age. Because the optimized assay used 10 μg/ml of total pupal protein as coating antigen, the percentage of total pupal protein of *M. raptorellus* larval origin that must be present for assay detection are 6.3% (21-day-old larvae), 3.1% (14-day-old larvae), 25% (7- to 8-day-old larvae), and ~100% (4- to 5-day-old larvae).

Artificial Nonemergent House Fly Pupae

Fifty-nine (59%) of 100 house fly pupae freeze-killed 14 days after *M. raptorellus* exposure were parasitoid ELISA-positive (Fig. 6, left column), whereas adult parasitoids emerged from 65 of the unfrozen pupae. There was no difference in the proportion of parasitoid-positive pupae by ELISA and parasitoid emergence (Yate's corrected $\chi^2 = 0.53$, $P = 0.47$).

TABLE 2

Global (Cutoff-Independent) Performance of *M. raptorellus* ELISA Based on Testing of 201 Negative-Reference (Unparasitized) and 427 Positive-Reference (*M. raptorellus*-Parasitized Larvae) Dipteran Pupae and Assay Data Expressed as Net Absorbance ($OD_{405/490}$)

Negative-reference dipteran pupae	Positive-reference house fly pupae	Area under ROC curve[a] (standard error)	*z* score (*P* value[b])	Overlap index
101 House fly and 100 stable fly	427 Pupa with 1- to 21-day-old larva	0.9486 (0.0079)	56.8 (<0.000001)	0.1028
101 House fly and 100 stable fly	327 Pupa with 7- to 21-day-old larva	0.9986 (0.0008)	656.1 (<0.000001)	0.0028
101 House fly	100 Pupa with 1- to 5-day-old larva	0.8266 (0.0290)	11.3 (<0.000001)	0.3468
101 House fly	107 Pupa with 7-day-old larva	0.9979 (0.0014)	360.4 (<0.000001)	0.0042
101 House fly	110 Pupa with 14-day-old larva	1.0000 (NA[c])	NA	0.0000
101 House fly	110 Pupa with 21-day-old larva	1.0000 (NA)	NA	0.0000

[a] ROC curve, receiver-operator characteristic curve.

[b] One-tailed *P* value (based on *z* score) for null hypothesis that ROC area = 0.5; $P < 0.05$ indicates that ROC area is statistically greater than 0.5 and the diagnostic test has significant overall discriminatory ability.

[c] NA, not applicable.

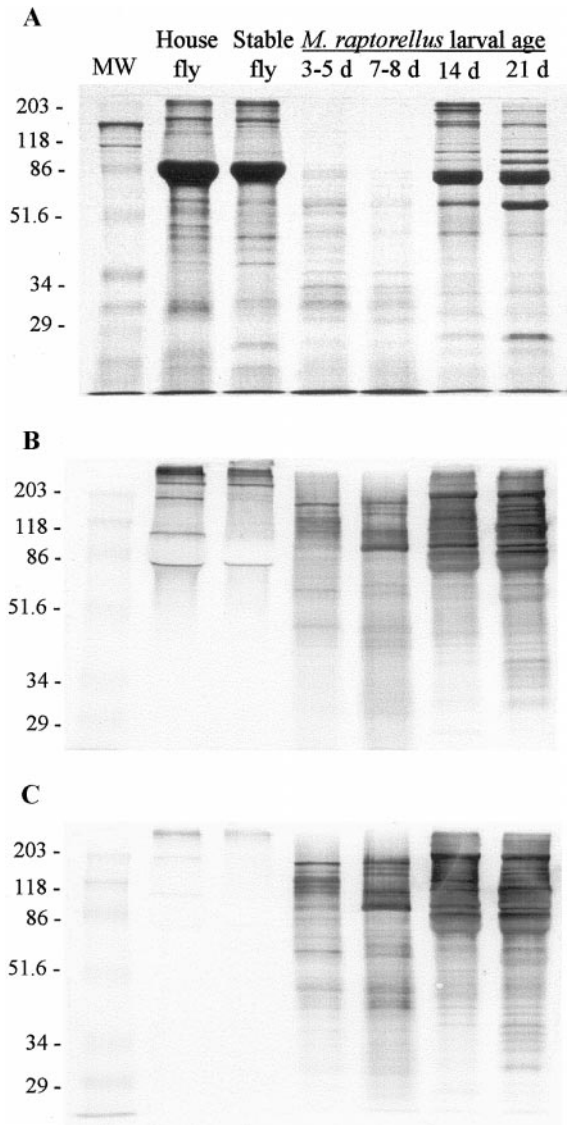

FIG. 3. SDS–PAGE and Western immunoblots of polyclonal rabbit anti-*M. raptorellus* larval antisera (from rabbit 6D; absorbed and unabsorbed, 1:2000 dilution) on dipteran host house fly and stable fly pupae (lanes 2 and 3) and on *M. raptorellus* larval parasitoid antigen of ages 3–5, 7–8, 14, and 21 days poststing (lanes 4 to 7, respectively) loaded at 35 μg protein per well. (A) Coumassie brilliant blue (protein)-stained gel; (B) immunoblot with unabsorbed *M. raptorellus*–immune rabbit sera (1:2000 dilution); (C) immunoblot with house fly pupal antigen-absorbed *M. raptorellus*–immune rabbit sera (1:2000 dilution). Lane 1, molecular weight markers (MW) in kDa.

Non-M. raptorellus Pteromalidae

The ELISA detected 45 of 50 (90%) 10- to 14-day-old microdissection-verified *M. raptor* larvae and 14 of 20 (70%) 10-day-old microdissection-verified *S. nigroaenea* larvae in house fly pupal hosts (Fig. 6). The mean $OD_{405/490}$ for the 50 *M. raptor*-parasitized pupae was 0.7915 (SD = 0.3585); the mean $OD_{405/490}$ for the 20 *S. nigroaenea*-parasitized pupae was 0.5392 (SD = 0.3826). These data suggest that there are many antigens common to *M. raptorellus*, *M. raptor*, and *S. nigroaenea* parasitoid larvae.

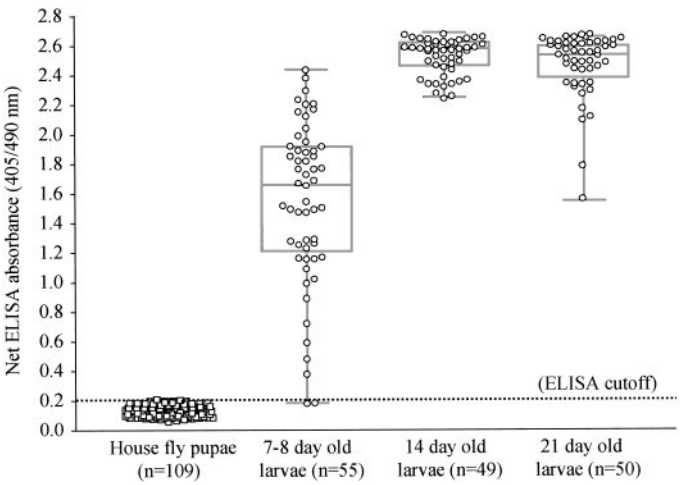

FIG. 4. Dot-box plots of net absorbance ELISA values for 109 unparasitized house fly pupae (n = 109) and 7-, 14-, and 21-day-old microdissection-verified *M. raptorellus*-parasitized house fly pupae (n = 154); all 263 house fly pupae were tested blindly (i.e., without knowledge of actual parasitized or nonparasitized pupal state) by ELISA. Dot-box plot interpretation is described in the legend to Fig. 1.

TABLE 3

Comparative Cross Classification, Test Performance, and Agreement between ELISA and Microdissection for Detection of *M. raptorellus* Larval Parasitoids of 7, 14, and 21 Days of Age in House Fly Pupae

Diagnostic assay cross classification	Microdissection		
	Positive	Negative	Totals
Parasitoid ELISA			
Positive[a]	152	2	154
Negative	2	107	109
Totals	154	109	263
Test performance[b]	Calculation	Estimate	95% CI[c]
Relative sensitivity	152/154=	98.7%	95.4–99.8
Relative specificity	107/109=	98.2%	93.5–99.8
False positive proportion	2/109=	1.8%	0.2–6.5
False negative proportion	2/154=	1.3%	0.2–4.6
Test agreement			
Concordance (accuracy)	259/263=	98.5%	96.2–99.6
Kappa[d]		0.969	0.938–0.999
McNemar's χ^2		0.250	(P = 0.617)

Note. All 263 fly pupae were tested blindly (i.e., without knowledge of pupal parasitoid status) by ELISA.

[a] Includes 7- to 8-day-old (n = 55), 14-day-old (n = 49), and 21-day-old (n = 50) larvae.

[b] Performance characteristics of ELISA relative to microdissection.

[c] CI, confidence interval.

[d] Kappa >0.75, excellent agreement beyond chance; kappa >0.40 and <0.75, good agreement beyond chance; kappa <0.40, poor agreement beyond chance.

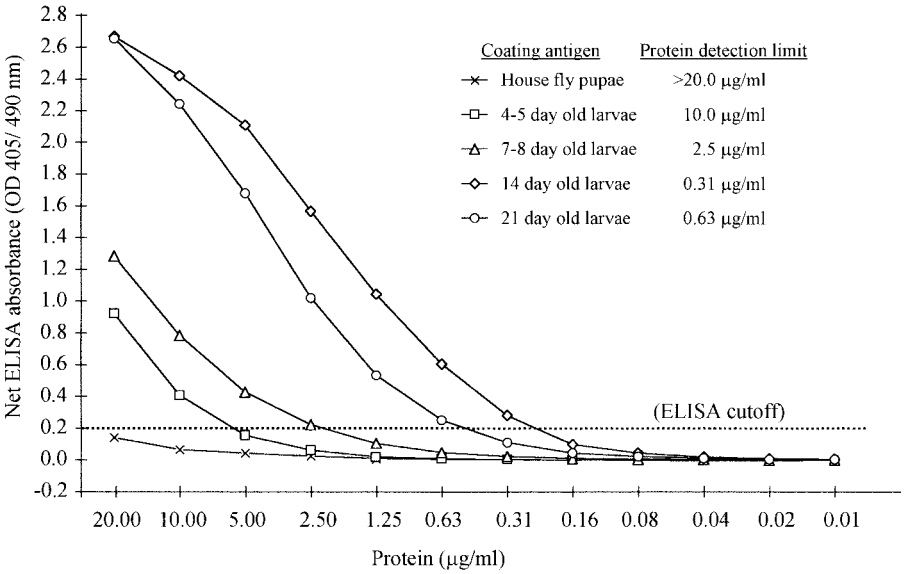

FIG. 5. Effect of *M. raptorellus* larval age on parasitoid ELISA protein detection limit.

Effect of Absorbed Versus Unabsorbed Immune Rabbit Serum

The beneficial effects of serum absorption on ELISA performance are shown in the detection limit titration curve in Fig. 7. At the optimized ELISA antigen coating conditions of 10 µg protein/ml, there was increased assay specificity with absorbed antisera and minimal sensitivity loss. The greater specificity of absorbed antiparasitoid antisera compared to that of unabsorbed antisera for *M. raptorellus* antigens in Western blot assay format is evident by comparison of Figs. 3B and 3C. In both the Western blot and the optimized ELISA, use of absorbed sera eliminated most cross-reactivity among host and parasitoid antigens.

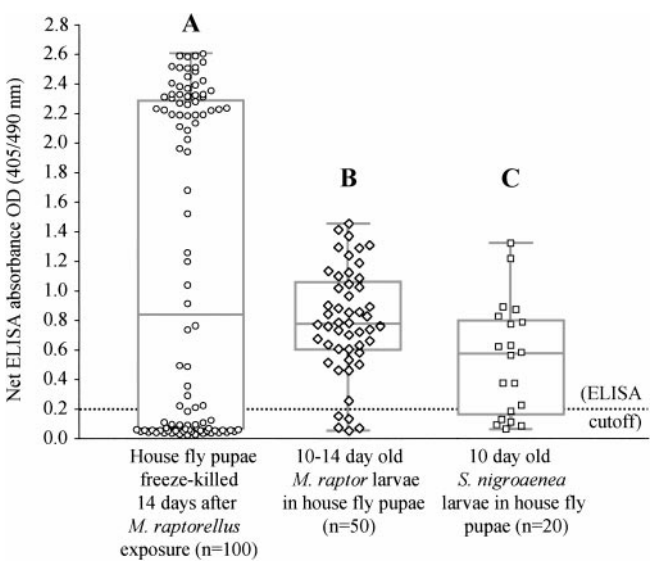

FIG. 6. Dot-box plots of net absorbance ELISA values in artificial nonemergent *M. raptorellus*-exposed house fly pupae and in house fly pupae parasitized (microdissection-verified) by larvae of non-*M. raptorellus* hymenopteran parasitoids. (A) *M. raptorellus* larval detection in freeze-killed fly pupae (14 days poststing) after room-temperature storage for 72 h (i.e., artificial nonemergent pupae); (B) *M. raptor* larval detection; (C) *S. nigroaenea* larval detection. Dot-box plot interpretation is described in the legend to Fig. 1A.

DISCUSSION

This report describes the development of a polyclonal antibody-based ELISA for the immunological detection of *M. raptorellus* larval parasitism in house fly pupae by rapid ELISA with Western blot confirmation. The ELISA technique has several potential advantages over parasitoid detection by adult wasp pupal emergence, adult fly pupal noneclosion (parasite-induced mortality), or pupal microdissection. First, early pupal parasitism of 7 to 14 days poststing, which is difficult to detect (and therefore easily missed) by microdissection, is sensitively detected by ELISA. Second, among noneclosed host pupae, the ELISA can at least partially distinguish parasitoid-induced mortality from other causes of pupal mortality. Because nonemergent host pupae may represent 50% or more of the total pupal population in field and laboratory studies, this represents an important and critical refinement in accurate measurement of biological control effects attributable to parasitoids. Whereas this report describes an ELISA for detection of the gregarious parasitoid *M. raptorellus* in house fly pupae, the approach should be transferable and applicable to many other host–parasite systems of plants or animals. A third advantage of the parasitoid ELISA is that it can be rapidly and easily performed by persons without microdissection or phylogenetic keying skills. The currently employed

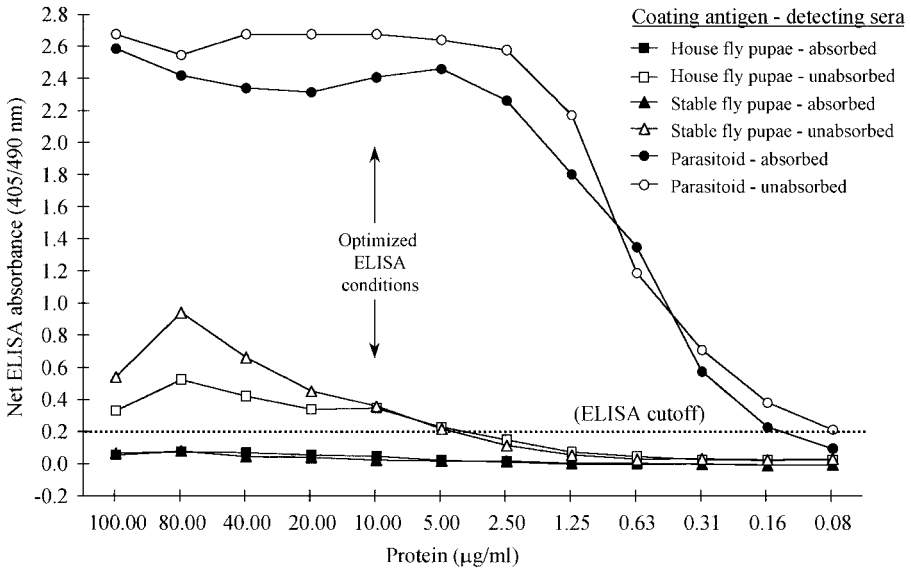

FIG. 7. Effect of serum absorption with house fly pupal antigens on *M. raptorellus* parasitoid ELISA detection limit (assay sensitivity for *M. raptorellus* antigen) and cross-reactivity with dipteran pupal antigens (assay specificity). The parasitoid antigen employed was pooled 7-, 14-, and 21-day-old macerated larvae.

methods of measuring pteromalid parasitism rates are known to be subject to error (Petersen and Currey, 1996a) and it is hoped that this ELISA procedure may lower this error rate if applied to field biocontrol studies.

The feasibility of immunoassay for detecting insect parasitism was demonstrated by the ELISA detection of parasitoids of stored-product pest insects (Stuart and Burkholder, 1991). Immunodetection of hymenopteran endoparasitoids within homogenized larvae of the polyphagous plant pests *Helicoverpa zea* (bollworm) and *Helithis virescens* (tobacco budworm) was also reported (Stuart and Greenstone, 1997). Immunodetection with various assay formats has been employed to analyze arthropod guts for prey remains (predator gut content immunoassays) (reviewed by Greenstone, 1996). Virtually all recent reports have advocated and employed monoclonal antibody (MAb)-based assays to minimize host–parasite or predator–prey antigen cross-reactivity and maximize specificity of the assay for the targeted parasite or prey genus and/or species. We employed absorbed polyclonal antibodies (PAb) to address this problem because absorbed PAb are much easier, faster, and less expensive to produce than MAb; PAb are also generally more sensitive for antigen detection than MAb. Nucleic acid-based techniques have also been applied to *Muscidifurax* spp. identification (Antolin *et al.*, 1996; Taylor *et al.*, 1997; Taylor and Szalanski, 1999). However, DNA-based molecular marker methods require greater technical expertise and more cost per sample tested and are much less amenable to large-scale population screening than is the ELISA method described here.

We recognize that whereas the ELISA accurately detected *M. raptorellus* larval parasitoids of 7 days of age or older, the assay did not adequately detect younger parasitoids. There are two potential problems in detecting young parasitoids. First, because these parasitic instars are naturally much smaller in mass than older parasites, they present a much smaller potential antigenic target for the polyclonal antiparasitoid antibodies. Second, the antigenic composition of developing parasitoid larval instars is in constant metamorphic quantitative and qualitative flux. This can be partially observed in the SDS–PAGE gel patterns (Fig. 3A) for the parasitoid larvae of different ages. The parasite antigen preparation used to immunize the rabbits in this study contained primarily parasitoid antigen derived from 14- and 21-day-old larvae, so that it is not entirely surprising that the sera did not contain a high titer of antibodies against unique younger larval epitopes. The protein detection limit curve (Fig. 5) for parasitoid larvae of various ages showed that a much higher absolute amount of parasitoid antigen was required from 3- to 5-day-old larvae (1.0 μg per assay well) than from 14- (0.03 μg per assay well) or 21- (0.06 μg per assay well) day-old larvae. This represents a 33-fold difference in antigen detection limit in 3- to 5-day-old larvae and 14-day-old larvae. Based on these data, generation of rabbit antisera with young larval parasitoids as immunogen may permit development of an ELISA that detects at least a proportion of these younger parasitoids. An alternative solution to this problem that does not require antisera against young larval parasitoids is development of a mathematical model of natural *M. raptorellus* parasit-

ization. By use of this solution, the total level of parasitoidism would be estimated by multiplication of the known level of parasitization due to 7-day-old or older larvae by an adjustment factor of greater than 1.0 to compensate for the proportion of true parasitization missed by the ELISA. Also, changing the immunoassay assay format may improve detection of young or first instar larval parasitoids in host insect homogenates. For example, Hagler (1998) reported improved detection of minute quantities of insect prey remains in insect predator guts by use of a "sandwich" or "capture" ELISA format compared to indirect or direct ELISAs. We are currently examining each of these possible solutions for improving our assay performance.

A critical step in assay development was use of antisera absorbed by house fly pupal antigens instead of nonhost-absorbed antisera. Serum absorption increased diagnostic sensitivity from approximately 90% to greater than 98% (data not shown) for pupae with larval parasites of 7 days of age or greater. The effect of serum absorption can be readily observed by comparison of the Western immunonblots with absorbed and nonabsorbed sera (Fig. 3) and by comparison of the ELISA protein detection limit curves for absorbed and nonabsorbed sera (Fig. 7). The Western blots with absorbed rabbit sera show nearly complete loss of cross-reactivity with house and stable fly host antigens, whereas the ELISA curve shows lack of cross-reactivity with either house or stable fly pupal antigens at up to 100 μg protein/ml, which is 10-fold higher than the coating antigen concentration of 10 μg/ml used in the optimized assay. Thus, use of absorbed sera largely accounted for the high diagnostic specificity of the ELISA. Serum absorption with potential cross-reacting antigens is a widely applied technique in bacterial serology that is used to improve the quality of diagnostic reagent antisera (Lindberg and Le Minor, 1984). Dempster (1960) used absorbed rabbit antisera to study predators of the broom beetle *Phytodecta olivacea* (Forster). However, most past attempts to employ absorbed polyclonal antisera in insect predator-gut assays have reduced the target detection limit to unacceptably low and ecologically useless levels (Greenstone, 1996). Whereas the ELISA described here did not show reactivity with host house or stable fly antigens, there was significant ELISA reactivity with the non-*M. raptorellus* parasitoid larvae of *M. raptor* and *S. nigroaenea* (Fig. 7). In theory, further absorption of antiserum with larval antigens from other pteromalids would increase the phylogenetic specificity of the ELISA for *M. raptorellus.* On the other hand, if it is considered more important to detect endoparasitism (by any species) than to identify a particular parasitoid species, then the ELISA reactivity with multiple pteromalids and species can be considered advantageous and desirable.

This study has demonstrated the potential of rapid PAb-based ELISA for immunologic detection of *M. raptorellus* larval parasitoids in house and stable fly pupae. For pupal parasitoid larvae which are at least 7 days of developmental age, the ELISA had diagnostic sensitivity and specificity exceeding 98%. Whereas we applied the rapid indirect ELISA technique to a specific host–parasitoid system of biological control interest in this study, the methods described here should be generalizable and applicable to many other insect or plant host–parasite or predator–prey systems for detection of minute beneficial or detrimental insects and/or parasites. In the future, use of (a) more highly purified antigens as immunogens, (b) more refined absorption of polyclonal antiparasitoid antisera, (c) other immunoassay formats, or (d) parasitoid-specific monoclonal antibodies in place of polyclonal antisera could further increase the diagnostic accuracy, ecological usefulness, and phylogenetic specificity of immunological-based parasite detection.

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